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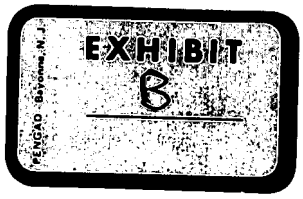
PATENT
Attorney Docket No.: A57660/DJB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Examiner: ZISKA
	)	
Weiss et al.	)	Group Art Unit: 1804
	)	
Serial No. 07/726,812	)	
	)	
Filed: July 8, 1991	)	
	)	
For: NOVEL GROWTH FACTOR RESPONSIVE PROGENITOR CELLS WHICH CAN BE PROLIFERATED IN VITRO	)	



DECLARATION UNDER 37 CFR 1.132

Commissioner of Patents
and Trademarks
Washington, DC 20231

Sir:

The undersigned, Brent A. Reynolds, hereby declares and states that:

1. I am a co-inventor of the subject application and I have read the arguments in the outstanding Official Action, dated May 5, 1993.

2. I am currently working on my Ph.D. at the University of Calgary. My Curriculum Vitae is attached hereto.

3. I have been asked to provide information as to the following:

I. Examples of the proliferation and differentiation of progenitor cells from species other than mice using the methods disclosed in the subject application;

II. Examples of the proliferation of progenitor cells in the presence of EGF or other growth factors at concentrations other than 20 ng/ml.

III. Examples of the proliferation and differentiation of progenitor cells from neural tissue other than that of the brain or striatum.

4. I have diligently performed and/or supervised experiments to demonstrate the proliferation of stem and progenitor cells, using substantially the same methods as those set forth in the subject application from species other than mice in the presence of EGF, as discussed in detail below in section I; at concentrations other than 20 ng/ml, as detailed in section II; and from tissue other than brain or striatum, as detailed in section III.

I. <u>Proliferation and differentiation of progenitor cells from species other than mouse</u>.

The data set forth below show that in addition to rodents, progenitor cells obtained from primates can be proliferated and differentiated using the methods disclosed in the subject application.

A. Proliferation and differentiation of adult monkey (Rhesus) progenitor cells.

Dissociation of cells: The conus medularis was removed from an adult male monkey (Rhesus) and hand cut with scissors into 1-mm sections and transferred into artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.35, approx. 280 mOsmol), aerated with 95% O_2 - 5% CO_2 at room temperature. After 15 min, the tissue sections were transferred to a spinner flask (Bellco Glass) with a magnetic stirrer filled with low-Ca^{2+} aCSF containing 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM

$NaHCO_3$, and 10 mM D-glucose (pH 7.35, approx. 280 mOsmol), aerated with 95% O_2 - 5% CO_2 at 32 to 35°C, containing 1.33 mg/ml of trypsin (9000 BAEE units/mg), 0.67 mg/ml of hyaluronidase (2000 units/mg) and 0.2 mg/ml of kynurenic acid. After 90 min, tissue sections were transferred to normal aCSF for 5 min prior to trituration. Tissue was transferred to DMEM/F-12 (1:1, Gibco) medium containing 0.7 mg/ml ovomucoid (Sigma) and triturated mechanically with a fire-narrowed pasteur pipet.

Methods for proliferation: Cells were plated (1000 viable cells per plate) in non-coated 35 mm culture dishes (Costar) containing serum-free medium (17) and 20 ng/ml EGF (human recombinant from Gibco/BRL).

Methods for differentiation: After 7 to 10 days in culture, floating spheres were transferred with wide-bore pipets onto laminin (15 μg/ml)(Sigma)-coated glass coverslips in 24-well culture dishes. EGF @ 20 ng/ml was added to the medium. Spheres attached to the substrate and cells within the sphere continued to proliferate.

Results: After 14 to 21 days in vitro (DIV), the cells were probed by indirect immunocytochemistry for the presence of neuron, astrocytes and oligodendrocytes. All three cell types were identified.

B. Proliferation and differentiation of embryonic human progenitor cells.

Dissociation of cells: With approval of the Research Ethical Committee at the University of Lund and the Ethics Committee at the University of Calgary, nine 8 - 12 week old human fetuses were obtained by suction abortions. Tissue was dissected and any identifiable brain regions were removed and placed in hibernation medium, packaged on ice and shipped, via air, to Calgary. Of the nine individual fetal brains that were sent, four were from eight week old

fetuses, four from nine week old fetuses and one from a twelve week old fetus. Upon arrival in Calgary (4 -5 days post-dissection), tissue pieces were mechanically dissociated into single cells with a fire-polished Pasteur pipette in serum-free medium composed of a 1:1 mixture of DMEM/F-12 and the number of viable cells counted.

Methods for proliferation: Cells were plated into 35 cm^2 tissue culture flasks (0.1×10^6 - 0.5×10^6 cells) with no substrate pre-treatment. The culture medium was composed of DMEM/F-12 (1:1) including HEPES buffer (5 mM), glucose (0.6%) sodium bicarbonate (3mM) and glutamine (2mM). A defined hormone and salt mixture composed of insulin (25 μg/ml) transferrin (100 μg/ml), progesterone (20 Nm), putrescine (60 μM) and sodium selenite (30 nM) was used in place of serum. To the above medium, 20 ng/ml of human recombinant EGF (Gibco/BRL) was added.

Two to three days after plating the cells, the majority of the viable cells had extended processes and had taken on a neuronal morphology. By seven days *in vitro* (DIV), the neuronal-like cells began to die and by 14 DIV nearly all of these cells were dead or dying (determined by the absence of processes, irregular membranes and granular cytoplasm). A few of the cells (1%) did not extend any processes or flatten nor did they take on an astrocytic morphology, instead these cells remained rounded and by 5 to 7 DIV began to divide. By 10 to 14 DIV, small clusters of cells, attached to the substrate, were identified. During the next 7 to 10 days (17 to 24 DIV), these small clusters continued to grow in size and many remained attached to the substrate. By 28 to 30 DIV, nearly all the proliferating clusters had lifted off the substrate and were floating in suspension. While floating in suspension, the clusters continued to grow in size and were passaged after they had been in culture for 30 to 40 days.

For passaging, floating spheres were collected, pelleted, supernatant removed, resuspended in 2 - 4 mls of DMEM/F-12 and mechanically dissociated with a fire polished glass Pasteur pipette. Dissociated cells were plated into untreated 35 cm^2 or 75 cm^2 tissue culture flasks with fresh culture medium and 20 ng/ml of EGF. EGF-responsive cells began to proliferate after a few DIV and formed floating spheres that were passaged a second time after 30 to 40 DIV.

Methods for differentiation: Thirty to 60 days after passage two or three, 2 - 3 ml aliquots containing media and pass 2 spheres were taken from the tissue culture flasks and plated onto 35 mm culture dish. Single spheres were placed onto poly-L-ornithine coated glass coverslips in DMEM/F-12/HM medium containing EGF. Spheres immediately attached to the substrate and within the first 24 - 48 hours cells begin to migrate from the sphere. At 14 DIV cells continued to proliferate and migrate resulting in an increase in the diameter of the transferred sphere. By 30 DIV, a large number of cells had been generated from the original sphere and had migrated at a similar rate from the center producing a concentric circle of associated cells. At the periphery, the majority of the cells were one cell layer thick while closer to the center there were denser regions of cells.

Results: Forebrain regions from eight week old tissue produced no spheres, while spheres were observed from hindbrain tissue in two of the four eight week old samples. For the nine week old fetuses, spheres were generated from forebrain region in two of the four samples and in two of the three hindbrain regions which were received. The twelve week old fetus contained only hindbrain tissue and spheres were produced.

Spheres generated from primary culture or pass 1 spheres were removed from the tissue culture flask, without inducing differentiation, and plated onto poly-L-ornithine coated glass coverslips in DMEM/F-12/HM medium for two hours to allow the spheres to attach to the substrate. Coverslips were removed and processed for indirect immunohistochemistry. Immunostaining with antibodies directed against neurofilaments (168 kDa) or GFAP did not identify any immunoreactive (IR) cells. However, nearly all of the cells were immunoreactive with an antibody that recognizes human nestin. Nestin is an intermediate filament protein found in undifferentiated neural cell. Therefore, EGF-responsive cells obtained from human tissue proliferate, generating clusters of undifferentiated cells that grow in suspension, can be dissociated, and replated, generating new spheres, composed of undifferentiated cells.

Thirty to 45 days after being plated onto the poly-L-ornithine coated substrate, cells were fixed and processed for indirect immunocytochemical analysis with antibodies directed against: MAP-2, Tau-1, neurofilament 168 kDA, GABA, Substance P (neuronal markers); GFAP (astrocyte marker); 04 and MBP (oligodendrocyte markers). Numerous MAP-2 and Tau-1-IR cell bodies and processes were identified in addition to a large number of Tau-1-IR fibers. While there was no indication of Substance P immunoreactivity, GABA-IR cell bodies with long branched processes were seen. Neurofilament-IR cells were strongly IR for GFAP. O4-IR cells with an O-2A morphology and an oligodendrocyte morphology were present. MBP-IR (found on oligodendrocytes) was also seen throughout the cultures.

II. Proliferation of progenitor cells in the presence of EGF at concentrations other than 20 ng/ml.

Methods: Embryonic day 14 (E14) CD_1 albino mice (Charles River) were decapitated and the brain and striata removed using sterile procedure. The tissue was mechanically dissociated with a fire-polished Pasteur pipette into serum-free medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (Gibco). The cells were centrifuged at 800 r.p.m. for 5 minutes, the supernatant aspirated, and the cells resuspended in DMEM/F-12 medium for counting.

The cells were suspended in a serum-free medium composed of DMEM/F-12 (1:1) which included glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), HEPES buffer (5 mM) and a defined hormone mix and salt mixture (to replace serum) that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) (all from Sigma except glutamine [Gibco]). In addition, the medium contained EGF (purified from mouse submaxillary, Collaborative Research) at 1, 10, 50, or 100 ng/ml. The cells were plated at 0.2 x 10^6 cells/ml into 75 cm^2 tissue culture flasks (Corning) with no substrate pre-treatment and housed in an incubator at 37°C, 100% humidity, 95% air/5% CO_2.

When the cells were proliferated, within the first 48 hours and by 3 to 4 DIV, they formed small clusters, known as neurospheres, that lifted off the substrate between 4 to 6 DIV.

After 7 DIV, the neurospheres were removed, centrifuged at 400 r.p.m. for 2-5 minutes, and the pellet was mechanically dissociated into individual cells with a fire-polished glass Pasteur pipet in 2 mls of complete medium.

1 x 10^6 cells were replated into a 75 cm^2 tissue culture flask with 20 mls of the EGF-containing complete medium. The proliferation of the stem cells and formation of new neurospheres were reinitiated.

Results: In all cases, i.e. at 1, 10, 50 and 100 ng/ml EGF, the rate of proliferation, number of cells generated, and morphological characteristics of the spheres were identical to that seen when 20 ng/ml EGF is used. When the cells were differentiated, neurons, astrocytes, and oligodendrocytes were identified immunocytochemically (as is the case when EGF at 20 ng/ml is used).

III. Proliferation and differentiation of progenitor cells from tissue other than that of the brain or striatum.

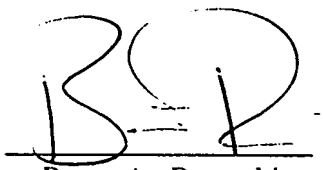
For the embryonic mouse, neurospheres have been generated (using the same technique as for the striatum) from the following neural regions: cortex, septum, thalamus, ventral mesencephalon and spinal cord. In all cases the neurospheres that were generated appeared to have the same characteristics as those generated from striatal tissue. The neurospheres generated from these regions also seemed to have the same differentiation potential in that they gave rise to neurons [some which contained GABA and substance P as their neurotransmitters], astrocytes and oligodendrocytes).

Additionally, EGF-responsive cells have been isolated from adult monkey conus medularis and proliferated and differentiated using substantially the same procedures as those disclosed in the subject application. The details of these procedures are set forth in Part A(4) above.

5. Based on the results set forth above, I conclude that by using the methods set forth in the subject application one can successfully proliferate and differentiate progenitor cells obtained from mammalian species other than mouse (e.g. human and monkey), from neural tissue at regions other than

brain/striatum (e.g. spinal cord, conus medularis), and using EGF concentrations other than 20 ng/ml (e.g. 1 ng/ml, 100 ng/ml).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that willful, false statements may jeopardize the validity/enforceability of the application or any patent issued thereon.

Dated: 2-11-93 Signature: 

Brent A. Reynolds